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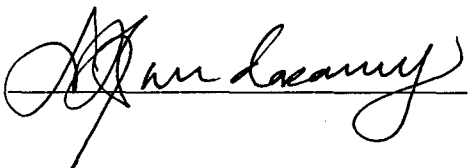
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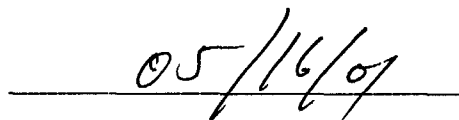
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13. ABSTRACT (Maximum 200 Words) Selective Estrogen Response Modifiers (SERMs) are a group of drugs that are currently being developed to kill breast cancer cells without inducing unwanted side effects, such as endometrial cancer. Several of the well established SERMs (such as tamoxifen) have been used clinically to treat patients with disseminated breast cancer, and more recently as a chemoprevention strategy in woman at high risk for breast cancer. We have looked at the mechanism by which these drugs induce tumor regression, and have shown that in cell culture, they stop the cancer cells from dividing and cause a significant percentage of the cells to die by a process called apoptosis. We have also shown that <i>in vitro</i> SERMs do not kill all the estrogen dependent MCF-7 cells. In fact, several of the SERMs (notably tamoxifen), induce a small proportion of the surviving cancer cells to become invasive. We have initiated <i>in vivo</i> experiments using genetically tagged cell lines to determine whether tamoxifen alters the metastatic load when used to treat orthotopically implanted hormone dependent breast cancer cells. Confirmation of the <i>in vitro</i> data would suggest that the decision of which SERM should be used for treatment of organ confined breast cancer or for chemoprevention of breast cancer should be based not only an assessment of the ability of SERMs to induce cell cycle arrest and cell death but also their potential for inducing metastatic behavior in the surviving cells.				
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TABLE OF CONTENTS

Cover.....	
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	12
Conclusions.....	16
References.....	16
Appendices.....	N/A

Final Report for Grant Number DAMD 17-97-7298

Covering Period September 1, 1998 to August 31, 2000

TITLE: APOPTOSIS AND TUMOR INVASION IN BREAST CANCER

INTRODUCTION

Selective Estrogen Response Modifiers (SERMs) are a group of drugs that are currently being developed to kill breast cancer cells without inducing unwanted side effects, such as endometrial cancer. Several of the well established SERMs (such as tamoxifen) have been used clinically to treat patients with disseminated breast cancer, and more recently as a chemoprevention strategy in woman at high risk for breast cancer. We have looked at the mechanism by which these drugs induce tumor regression, and have shown that in cell culture, they stop the cancer cells from dividing and cause a significant percentage of the cells to die by apoptosis. We have also shown that in the estrogen dependent breast cancer cell line we have used (MCF-7) SERMs do not kill all of the tumor cells. In fact, with several of the SERMs (including tamoxifen), a small proportion of the surviving cancer cells become invasive. This acquisition of invasive behavior is of concern since it is the first step in the process of metastasis. To study the progression to a metastatic phenotype we have developed several estrogen dependent MCF-7 sublines that are genetically tagged and a parallel set of sublines derived from the estrogen independent SUM 159PT cell line. These cell lines are being used to initiate a series of studies designed to directly examine the metastatic progression of breast cancer in an orthotopic implantation model of breast cancer. The specific findings and how they relate to the revised statement of work is outlined below in the body of this report.

BODY

The body of this application contains the revised statement of work for this award with an indication of the status of the project and problems encountered in the execution of several tasks, followed by a more detailed summary of the results

Specific Aim 1: Characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro

Task 1 (months 1-4): Develop time and dose dependence of acquisition of invasive phenotype of parental MCF-7 cell line [**status: completed**]

Task 2 (months 12-18): Characterize changes in gene expression and DNA fragmentation associated with the acquisition of the invasive phenotype in parental MCF-7 cells. [**status: completed**]. RNA and protein have been extracted from the parental cell line, and the level of several transcripts, including clusterin, cathepsin B and uPA have been assessed by RT-PCR. The protein levels have been assessed by Western analysis. DNA fragmentation remains to be assessed by gel electrophoresis and by TUNEL staining.

[status completed]

Task 3 (months 2-8): Develop clonal MCF-7^{ae}_{inv} sublines of invasive cells. **[status: completed]**. We have isolated and clonally selected a number of MCF-7^{ae}_{inv} sublines, and have demonstrated that some, but not all, of the cell lines have retained sensitivity to ICI 182,780.

Task 4 (months 8-12): Characterize changes in gene expression associated with stable acquisition of invasive phenotype in MCF-7^{ae}_{inv} sublines. **[status: completed]**. We have isolated RNA and protein from several of the MCF-7^{ae}_{inv} sublines and level, and we have performed RT-PCR and Western analyses to establish the changes in the levels of the relevant transcripts and proteins in the invasive sublines.

The results from Tasks 1-4 of specific Aim 1, which are summarized below are currently being written up in manuscript form for submission to *Breast Cancer Research and Treatment*.

Task 5 (months 12-15): Characterize the sensitivity of MCF-7^{ae}_{inv} to TNF α and anti-estrogens (tamoxifen, 4-hydroxytamoxifen and ICI 182,780) **[status: partially complete]**. We are comparing the sensitivity of these cell lines to other anti-estrogens (tamoxifen and 4-hydroxytamoxifen) to identify cell lines that have retained their sensitivity to anti-estrogens and others that have developed resistance to the drugs. There appear to be several different sensitivity profiles, and we are still cataloging these profiles to determine whether there is an underlying biological explanation for these differences that can be attributed to estrogen receptor levels or the ability of the cells to initiate cell death.

Specific Aim 2: Compare the sensitivity of the MCF-7^{ae}_{inv} sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis

Task 1 (months 12-18): Characterize the effects of EB1089 and onapristone on apoptosis and invasion in parental MCF-7 cells compared to MCF-7^{ae}_{inv} sublines. **[Status: completed, as far as possible]**. Our results demonstrate that the parental MCF-7 cells undergo apoptosis in response to EB1089 and that there is no measurable induction of invasion by this vitamin D analog. As a result it has not been possible to develop MCF-7^{D3}_{inv} sublines. The effects of onapristone have not been investigated since the company that manufactures the anti-progestin (Schering AG) has removed it from the market, and will not sanction its use as an anti-cancer therapy. In light of the demonstration that EB1089 is capable of killing MCF-7 cells without inducing an invasive phenotype, and is also effective in killing the invasive cell lines we have developed, we have decided not to pursue similar experiments with the an alternative anti-progestin such as RU-486, but to concentrate on the biology underlying the effects of the vitamin D analogs.

Task 2 (months 12-18): Characterize the effects of simultaneous administration of anti-estrogen and EB1089 or onapristone on apoptosis and acquisition of the invasive phenotype on parental MCF-7 cells. Characterize the changes in gene expression and

DNA fragmentation. **[Status: partially completed]**. As stated above, this work has concentrated only on the effects of the vitamin D analogs.

Task 3 (months 15-24): Develop clonal MCF-7^{ona}_{inv} and MCF-7^{D3}_{inv} and compare their sensitivity to anti-estrogens and TNF α . **[Status: abandoned]**. Since we have been unable to obtain invasive MCF-7 sublines after treatment with EB1089, it appears that vitamin D analogs do not induce an invasive phenotype. Work with anti-progestin (onapristone) has been abandoned because we could not ensure a continued supply of the drug, particularly for future *in vivo* studies.

Task 4 (months 12-24): Determine degree of cross sensitivity for each invasive cell type and characterize changes in gene expression. **[Status: partially completed]** Preliminary results, that need to be repeated have suggested that most MCF-7^{ae}_{inv} sublines are sensitive to the synthetic vitamin D analog EB1089, and the majority of the cell die after treatment with this drug.

As we have pointed out previously, prior to the initiation of the research we were asked to describe how we would pursue this work in vivo as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two year funding period.

Specific Aim 3: *To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo. To perform these experiments we modified our experimental timetable to include an additional preliminary task: the development of the development of estrogen sensitive clonal cell lines expressing enhanced Green Fluorescence Protein (GFP). We have therefore developed a number of MCF-7^{GFP} sublines, and have compared the effects of anti-estrogens on these breast cancer cell lines to ensure that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens. **[status completed]**. These cell lines are currently being analyzed in parallel with the parental MCF-7 cells, we are isolating MCF-7^{GFP} that are also invasive, and we are characterizing the sensitivity of these fluorescent sublines to anti-estrogens and TNF α . We have identified several MCF-7^{GFP} sublines that has the same characteristics as the parental line so that we can perform the in vitro studies proposed in specific aim 3, using thoroughly tested, appropriate cell lines in these latter experiments. We have tested these cell lines in parallel with the parental MCF-7 cells and have isolated MCF-7^{GFP}^{ae}_{inv} cell lines comparable to those we have derived from the parental line. **[Status: partially completed]** Estimated revised time frame: These experiments should be completed between months 36-40. This timeline extends beyond the 3 year funding period, but as we have pointed out in our initial description of specific aim 3 and the progress report, we understand that funding for the *in vivo* studies which would be initiated*

after month 36, has not been secured, and is not guaranteed.

Effect of Move on the Project:

The transfer of our laboratory from the Adirondack Biomedical Research Institute (formerly the W. Alton Jones Cell Science Center) in Lake Placid to the Department of Biological Sciences at the University of Notre Dame, and the delay in transferring the grant to the new institution resulted in an 8 month hiatus. This coupled with the no-cost extension from the university, put us on target to complete the research in June, 2000. This effectively puts the project, which was originally planned as a two year program, in synchrony with the three year grants awarded by the DAMD in 1997. The data presented in this report was presented at the Era of Hope Meeting in Atlanta Georgia in June 2000.

Other than the downtime, we have not experienced any adverse effects on the project resulting from the move. We have re-established each of the cell lines in culture since we moved and have demonstrated that the parental MCF-7 and the MCF-7^{GFP} cell lines have not been affected by the move. The facilities available in our new laboratories, which were constructed to our specifications, are considerably superior to the facilities we left in Lake Placid. A number of new instruments including superior microscopes and electronic image documentation and analysis for microscopy and gel electrophoresis (Western and RT-PCR) has significantly improve our throughput, making it possible to catch up for lost time.

In the original application there were two specific aims:

Specific Aim 1. *To characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro.*

Specific Aim 2. *To compare the sensitivity of MCF-7^{ae}_{inv} sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis.*

Progress since Re-initiation of the Grant

We have compared the effects of several anti-estrogens to TNF α on MCF-7^{WT} cells. We have established that the anti-estrogens induce cell cycle arrest and apoptosis of MCF-7^{WT} cells in a dose manner (Fig. 1). These data demonstrate that the MCF-7^{WT} display differential sensitivities to the individual anti-estrogens. Of the anti-estrogens tamoxifen and 4-hydroxytamoxifen appear to be considerably more potent (with an LD₅₀ of approximately 0.1 μ M), than the pure anti-estrogen, ZM 182, 780 (LD₅₀ of 2 μ M). Time course analysis of the effects of selected doses of each anti-estrogen demonstrates that

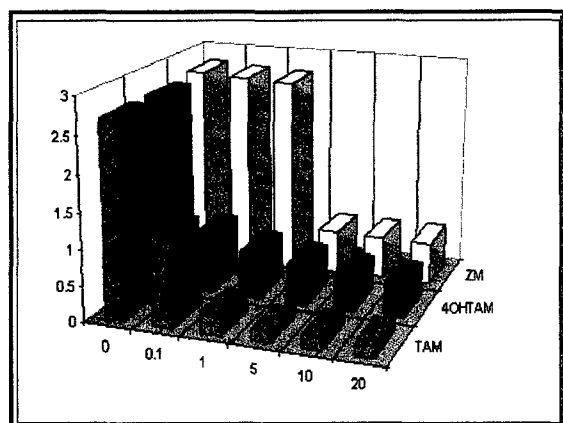


Figure 1: Dose response of MCF-7WT cells to various anti-estrogens. The dose range was from 0.1 to 20 μ M. The cells were plated in phenol red free α -MEM, with 10% FCS and treated with the anti-estrogens for 72 h. The surviving cell numbers were determined by crystal violet staining. Results represent mean of 3 independent experiments.

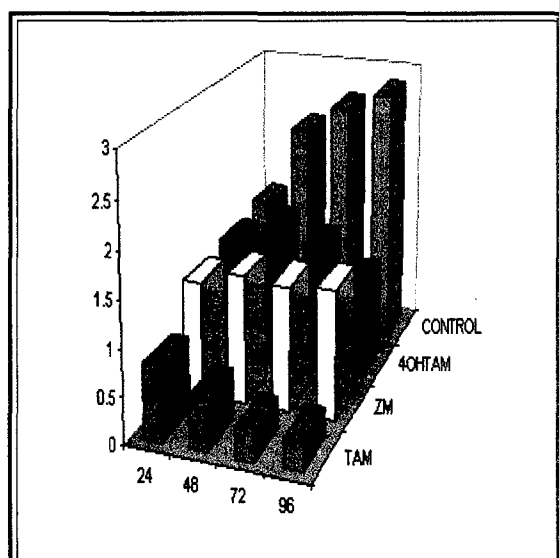


Figure 2: Time course of effects of various anti-estrogens on MCF-7^{WT} cells. Equal numbers of cells were plated in α -MEM and treated with 1 μ M tamoxifen, 4-hydroxytamoxifen or 10 μ M ZM 182,780. The cell numbers were determined by crystal violet at the indicated times. Results are the mean of 3 independent experiments

1 μ M tamoxifen induces a clear reduction in cell number, while 1 μ M 4-hydroxytamoxifen and 10 μ M ZM 182,780 block the increase in cell numbers seen in the control, untreated cells (Fig. 2).

Flow cytometry studies have shown that the effects of all three anti-estrogens is due to a combination of cell cycle arrest and apoptosis (data not shown). Regardless of the dose and time of treatment it is important to recognize that a significant number of cells survive the anti-estrogen treatments.

We have now demonstrated that a sub population of MCF-7^{WT} that survive anti-estrogen therapy acquire an invasive phenotype after treatment. In preliminary experiments we have established the appropriate parameters for measuring the ability of the MCF-7^{WT} and MCF-7^{GFP} cells to invade through Matrigel in a modified Boyden chamber assay, by monitoring cell number (measured by crystal violet staining) or intrinsic GFP fluorescence as they pass through the Matrigel and membrane of the chamber before and after treatment with anti-estrogens. As shown in Figure 3, MCF-7^{WT} cells do not invade through 8 μ invasion filters coated with a thick layer of Matrigel in the absence of the anti-estrogens. However after treatment with tamoxifen or 4OH-tamoxifen, a sub population of the surviving cells acquire an invasive phenotype. Treatment of the MCF-7^{WT} cells with TNF α or ZM 182,780 (which also induce cell death), does not induce an invasive phenotype in the surviving cells. It is clear that treatment with tamoxifen or 4-OH tamoxifen increases the invasive potential of the MCF-7 cells to a level that is equal to or exceeds that of the SUM159T cells, a breast cancer cell line that we have

shown is invasive *in vitro* and metastatic *in vivo*. As Figure 4 demonstrates the acquisition of the invasive phenotype is enhanced by the chemotatic attractants in fibroblast-conditioned medium (presumably growth factors such as basic FGF and FGF-9), but the invasive cells do not require the presence of the conditioned medium to manifest the

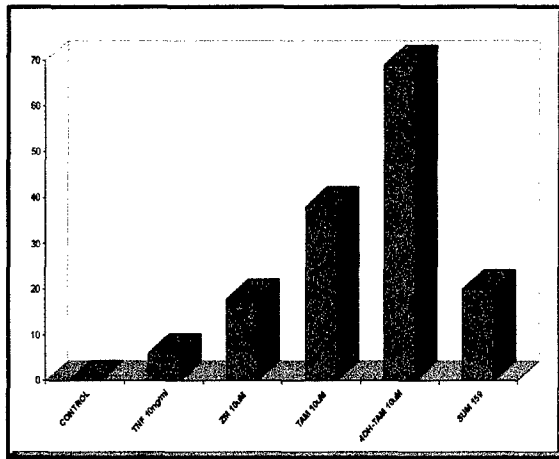


Figure 3: Induction of an invasive phenotype in MCF-7^{WT} cells treated with TNF α and various anti-estrogens. Equally numbers of MCF-7^{WT} cells were plated in each well, and treated with TNF α , ZM 182, 780 tamoxifen or 4-OH tamoxifen for 72 h. Surviving cells were collected and equal numbers were plated on 8 μ membranes coated with Matrigel. Cell numbers in the lower chambers were determined 48 h later by crystal violet. The data were compared to the level of invasion using a known metastatic cell line (SUM 159T cells).

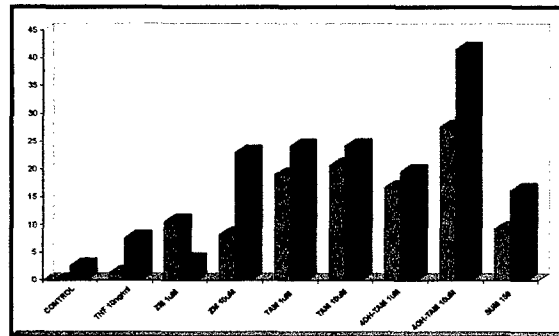


Figure 4 Effect of fibroblast-conditioned medium in the acquisition of the invasive phenotype. MCF-7^{WT} cells were grown, and treated with the indicated anti-estrogens as described in figure 3. Equal numbers of cells were plated on 8 μ membranes coated with Matrigel. The lower chamber contained either α -MEM (blue) or fibroblast conditioned medium (purple). Cell numbers in the lower chambers were determined 48 h later by crystal violet. The data were compared to the level of invasion using a known metastatic cell line (SUM 159T cells).

phenotype.

These studies confirm that a small sub-population of cells that survive anti-estrogen therapy become invasive. We have used RT-PCR to demonstrate that tamoxifen treatment of this subline induces the synthesis of MMP-2 and MMP-9 and cathepsin B, while down-regulating the synthesis of at least one inhibitor of the matrix metalloproteases (TIMP-1) and cystatin C. We are currently repeating these experiments to confirm these observations and to determine which other extracellular proteases (and acid hydrolases) are induced after treatment with anti-estrogens.

Using the vitamin D analog EB1089 we have confirmed our earlier observation that MCF-7 cells are sensitive to vitamin D and undergo apoptosis (Van Weelden et al., 1998). However to date we have not been able to demonstrate that this vitamin D analog induces an invasive phenotype in the surviving population of MCF-7 cells. In this respect the vitamin D analog appears to behave more like the pure anti-estrogen, ICI 182,780 and may therefore serve as an excellent adjuvant therapy for women being treated with tamoxifen for primary breast cancer, since it appears to induce apoptosis in many estrogen dependent breast cancer cells without initiating an invasive phenotype and may even kill some estrogen sensitive cells that have abrogated apoptosis and induced invasion.

Prior to the initiation of the research we were asked to describe how we would pursue this work in vivo as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two funding period.

Specific Aim 3: *To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of*

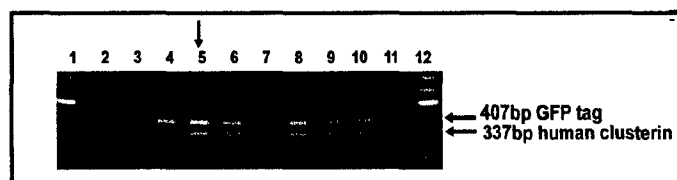


Figure 5: PCR based analysis of stable transfection of pEGFP-c1 into MCF-7 cells. Lanes 1 and 12: 100 bp ladder. Lanes 2-11: individual clones expressing GFP. Comparison of the GFP signal to that of clusterin (which is present in 2 copies per cell) indicates that most cells contained between 2 and 4 copies of GFP per cell. Lanes 2 and 7 represent unsuccessful clonal expansion of the selected transfectants. The clone in lane 5 indicated by the arrow was selected for further studies as outlined below.

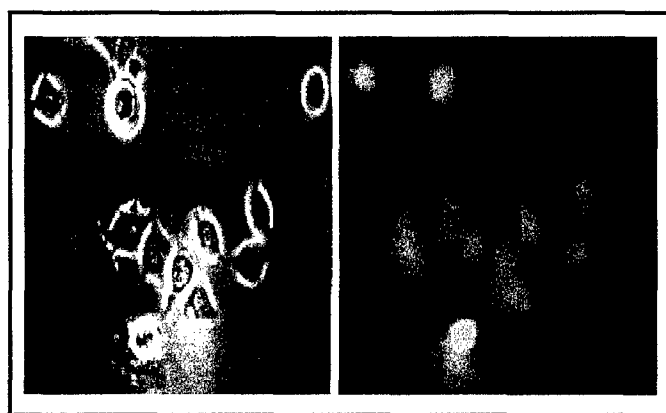


Figure 6: Phase contrast (left panel) and fluorescence microscopy (right panel) of MCF-7 cells transfected with a plasmid expressing green fluorescent protein (GFP). MCF-7^{GFP} cells were grown for 72 h in serum free defined medium prior to photography.

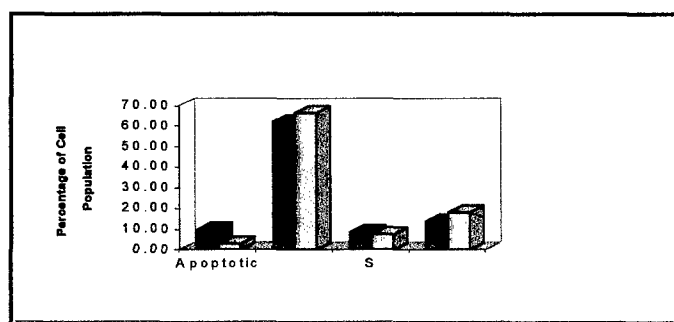


Figure 7
Comparison of cell cycle kinetics between parental MCF-7^{WT} cells (purple) and MCF-7^{GFP} (green). Cells were stained with propidium iodide and analyzed on a Coulter Epics flow cytometer.

[This page contains unpublished data]
metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo.

While specific aim 3 was clearly not part of the original application, our proposal to use MCF-7 cells transfected with GFP (green fluorescent protein) to monitor invasive cells both *in vitro* and *in vivo* suggested to us that we would be well advised to create these MCF-7^{GFP} sublines before proceeding to examine the effects of anti-estrogens on these breast cancer cell lines to ensure that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens.

We therefore modified our original experimental timetable to include an additional task: the development of estrogen sensitive clonal cell lines expressing enhanced Green Fluorescence Protein (GFP). These cell lines have been developed by transfecting estrogen dependent MCF-7 cell with pEGFP-c1, a plasmid containing the enhanced green fluorescent protein under the control of the constitutive CMV promoter, and an ampicillin selectable marker. These cells were cultured for 7 days, and then sorted on a Becton-Dickinson fluorescence activated cell sorter into 96-well culture plates using the autosort facility of the machine. The cells in individual wells were clonally expanded to produce a series of high, medium and low GFP-expressing sublines by growth in G418. A total of 20 sublines have been stabilized,

characterized and frozen in liquid nitrogen for further use. The PCR based analysis of 10 of these cell lines is shown in Fig. 5, and demonstrates that for most of these lines,

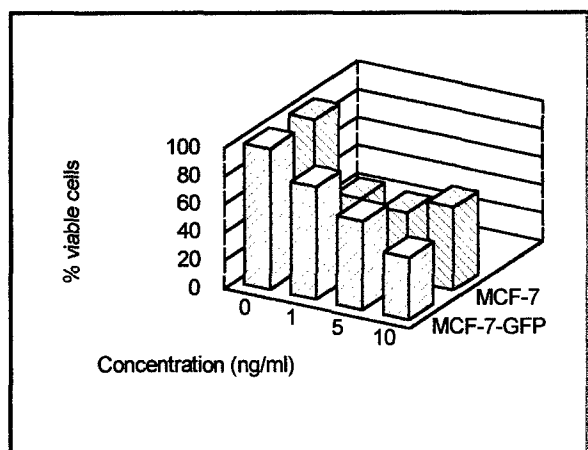


Figure 8: Comparison of sensitivity of MCF-7^{GFP} and parental MCF-7^{WT} to increasing doses of TNF- α . The two cell lines were grown for 4 days and then treated with increasing doses of TNF- α . After 72 h the number of surviving cells was estimated using crystal violet, and compared to untreated controls. Results are the mean of three independent experiments.

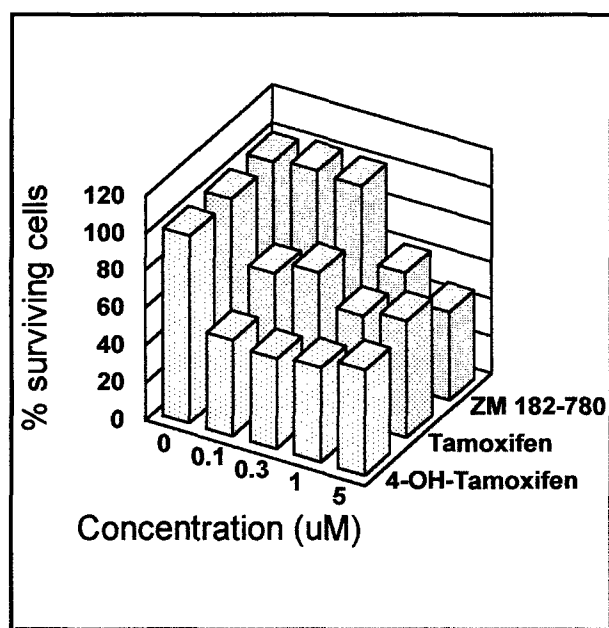


Figure 9: Dose response of MCF-7^{GFP} cells to ZM182,780, tamoxifen or 4-hydroxytamoxifen. MCF-7^{GFP} cells were incubated with each of the indicated anti-estrogens for 72h. The cells were harvested and the cell viability was assessed using crystal violet and compared to untreated cells incubated for the same length of time. Results are average of two independent experiments.

[This page contains unpublished data] clusterin is present at between 2 and 4 copies per cell. Phase contract and fluorescence photomicrographs of the subline represented in lane 5 is shown in Figure 6, demonstrating that virtually all of the cells express equivalent amounts of GFP.

In our initial experiments we have established that transfection of the MCF-7 cells with the GFP construct has not significantly affected the cell cycle kinetics (Fig. 7). Both MCF-7^{WT} and MCF-7^{GFP} have essentially the same proportion in each phase of the cell cycle, suggesting that the GFP transfection has not altered the cell cycle kinetics. We have also compared the sensitivity of this subline to TNF- α with that of the parental MCF-7 wild type cells (MCF-7^{WT}). As shown in Fig. 8, the MCF-7^{GFP} retain their sensitivity to TNF- α and demonstrate a very similar dose response to the drug as the parental wild type MCF-7 cells, suggesting that the expression of GFP in these cells does not influence the ability of the transfected cells to respond to TNF α and undergo apoptosis after the appropriate stimulation (Fig. 8).

Most, but not all of the transfected sublines have also retained their sensitivity to anti-estrogens and induce cell death (as monitored by DNA fragmentation (TUNEL positivity) and cell viability as monitored by crystal violet or flow cytometry. The relative sensitivity of the subline shown in Fig 5, 6, and 7, to ZM182,780, tamoxifen and 4-hydroxytamoxifen is shown in Fig. 9. The results demonstrate that this particular subline retains its sensitivity to all three anti-estrogens, and like the parental MCF-7 cell line appears to be sensitive to the pure anti-estrogen ZM 182,780 only at higher concentrations (> 1 μ M), even though the

overall rate of cell death appears to be of the same order of magnitude (approximately 40% of the cells appear to survive anti-estrogen therapy for 72h). Most of the sublines

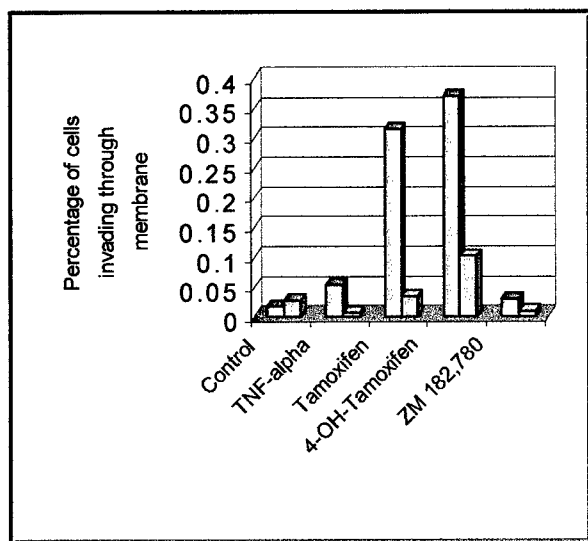


Figure 10: Induction of the invasive phenotype in MCF-7^{WT} (purple) and MCF-7^{GFP} (green) cells by anti-estrogens. Results are the mean of three independent experiments

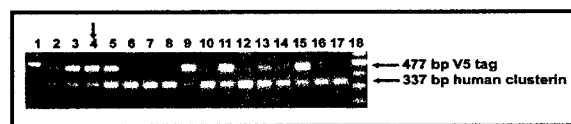


Figure 11: PCR based analysis of stable transfection of pV5-HisA into SUM 159PT cells. Lanes 1 and 18: 100 bp ladder. Lanes 2-17: individual clones expressing V5 tag. Comparison of the V5 signal to that of clusterin (which is present in 2 copies per cell) indicates that most cells contained between 2 and 4 copies of V5 per cell. Lanes 6, 7, 8, 10, 12, 16 and 17 represent unsuccessful clonal expansion of the selected transfectants. The clone in lane 4 indicated by the arrow was selected for further studies as outlined below.

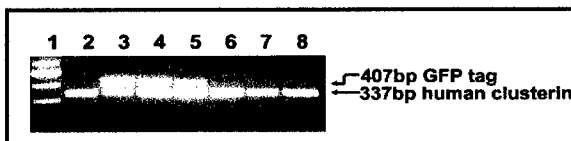


Figure 12: Demonstration of Sensitivity of PCR detection of MCF-7^{GFP} cells. MCF-7^{GFP} cells were mixed with increasing numbers of control cells not containing the GFP construct. After extracting DNA from the mixed cell population the DNA was extracted and level of GFP determined by PCR amplification. Lane 1: 100 bp ladder; Lane 2: SUM159 PT alone; Lane 2 MCF-7 GFP alone. Lane 4-8 SUM 159PT cells and MCF-7^{GFP} cells mixed in ratios of 1:5, 1:50, 1:500, 1:5,000 and 1:10,000. The GFP signal is readily detectable in mixes that contain 1 GFP containing cell in 5,000 cells.

display similar sensitivities to the individual anti-estrogens although we have not yet determined whether the (minor) variations we see are reproducible or biologically significant.

Importantly, both tamoxifen and 4OH tamoxifen induce an invasive phenotype in the MCF-7^{GFP} as they do with the MCF-7^{WT} cells (Fig. 10), while the pure anti-estrogen ICI 182,780 does not. This suggests that these tagged cells are suitable for the *in vivo* studies outlined in specific aim 3.

For comparative purposes in the *in vitro* invasion assays described in specific aim 1 and 2 and for the *in vivo* analysis of metastases described in specific aim 3 we have also developed a tagged estrogen independent, metastatic cell line - the SUM159PT cell line which we have stably transfected with the pcDNA6/V5-HisA tag. These cells have been clonally selected as described above for the MCF-7^{GFP} cells, and one subclone-number 4 (as indicated by the arrow) has been chosen for further study. Since these cells are invasive and metastatic they serve as control cell lines for both the *in vivo* and *in vitro* experiments. While the SUM 159PT cell lines are not fluorescent, we can monitor metastatic spread of these cells by PCR, using primers specific for the V5-HisA tag.

We have recently (August 20/2000) initiated a pilot study as part of a larger study funded by the Coleman Foundation, and approved the University of Notre Dame IACUC. The pilot study, using 25 mice, is designed to determine whether treatment with tamoxifen induces metastasis in MCF-7^{GFP} cells orthotopically implanted in the mammary fat pads of ovariectomized mice supplemented with estradiol. The estradiol supplementation is in the form of implantable pellets from Innovative Research that provide a physiological blood level of estradiol

(between 150-200 pg/ml). The primary tumors will be allowed to grow until they reach 1cm³ (approximately 3 weeks), at which time 20 animals will be implanted with tamoxifen pellets designed to sustain a level of 30-40 ng/ml, a level that is approximately 100 fold higher than the circulating estradiol and which we have previously shown induces tumor regression in the wild type cells. The remaining 5 animals will serve as control animals and will be sacrificed when the tumors reach 2cm³ or the animals show signs of discomfort. At monthly intervals we will sacrifice 4 animals, excise the primary tumors, if visible, as well as the lungs, liver, lymph nodes, brain and bone. These tissues will be inspected under a dissecting microscope for evidence of green fluorescence (indicative of metastatic MCF-7^{GFP} cells in tissues other than the primary tumor). In case the protein is being expressed at undetectable levels, or has ceased to be fluorescent, the tissue samples will also be analyzed by PCR for the presence of the GFP gene. As shown in Fig. 12, using this PCR based methodology we can detect 1 MCF-7^{GFP} cell in 500-5000 non-transfected cells, using 30 cycles of PCR. Thus we will be able to detect the presence of MCF-7^{GFP} cells in any macroscopic metastases that develop during or after tamoxifen treatment. The isolation and microdissection of small metastases has been made much more straightforward in the last month by the departmental purchase of an Arcturus Laser Capture Microdissection station. This will allow us to selectively dissect only tumor cells from metastatic sites (either based on fluorescence or on H&E staining) thus drastically reducing the contamination from host tissues. This pilot study will determine whether tamoxifen, in addition to inducing an invasive phenotype *in vitro* is also capable of inducing a metastatic phenotype *in vivo*. These studies will be completed by the end of 12/00.

If this pilot experiment demonstrates that tamoxifen induces metastatic progression (as monitored by the presence of GFP in distant sites), we will initiate a considerably expanded study to compare the effects of tamoxifen, 4-OH tamoxifen and ICI 182,780 alone and in combination with EB1089 on the induction of the metastatic phenotype in MCF-7 cells, and we will also determine whether these compounds alter the invasive and metastatic phenotype of the estrogen-independent SUM-159PT cell line, using the tagged cell lines described above and listed below.

KEY RESEARCH ACCOMPLISHMENTS

- ▶ demonstration that tamoxifen, but not ICI 182,780 or TNF α , induces an invasive phenotype in estrogen dependent MCF-7 breast cancer epithelial cells
- ▶ characterization of the induction in ECM protease expression in MCF-7 cells that survive tamoxifen therapy
- ▶ development of MCF-7 cell lines genetically tagged with GFP, V5/HisA and myc/HisA, and characterization of the effects of SERMs on selected sublines
- ▶ initiation of *in vivo* study to determine whether tamoxifen induces metastatic progression using the tagged cell lines described above

REPORTABLE OUTCOMES

1. Manuscripts:

There are two manuscripts currently in preparation describing the research outlined in the above report:

Louise Flanagan, Yao Wang, Ian Byrne, JoEllen Welsh and Martin Tenniswood. *Utilization of Green Fluorescent Protein for the Identification of Metastasis in an in Vivo Model of Breast Cancer Progression*. In preparation for Breast Cancer Treatment and Research.

Yao Wang, Louise Flanagan, Jacintha O'Sullivan, JoEllen Welsh and Martin Tenniswood. *Co-operation Between Invasive and Non-invasive Breast Cancer Cells During Metastatic Progression*. In preparation for International Journal of Cancer.

2. Abstracts:

Jacintha O'Sullivan and Martin Tenniswood. *Apoptosis and Tumor Invasion in Breast Cancer*. Poster presentation at the Era of Hope Meeting, Atlanta, Georgia June 2000.

Martin Tenniswood, Jacintha O'Sullivan, Kerry Gilmore and Edmund Lee. *Induction of Invasion as a Consequence of Abrogated Apoptosis*. Walther Cancer Institute Annual Retreat, University of Notre Dame, August, 2000.

3. Presentations

The following invited presentations have described the work supported by this award:

Relationship Between Apoptosis and Metastasis in Breast Cancer. Indiana University Cancer Center, Indianapolis, Indiana, May 1999.

Clusterin Biogenesis and ER Overload, a New Mechanism of Cell Death in Hormone Dependent Breast Cancer Cells. Keynote Address, Gordon Conference on Cell Death, Colby Sawyer College, New Hampshire, July 1999.

Development of Anti-estrogens to Improve the Treatment of Breast Cancer. Southern Great Lakes Local Section of the Society for Industrial Microbiology, Alpharma, Chicago Heights, Illinois October 1999.

Biogenesis of Clusterin and ER Overload in Cell Death. Department of Urology, Northwestern University, Chicago, Illinois, November 1999

Clusterin Biogenesis, Apoptosis and Tumor Progression in Breast Cancer. Center for Reproductive Biology, Washington State University, Pullman, WA, March 2000

4. Development of Cell Lines

We have developed a series of stably transfected cell lines that can be used to monitor metastatic spread of tumor cells that have been orthotopically injected into the mammary fat pad of nude mice. These cell lines include:

MCF-7 GFP
MCF-V5/HisA
MCF-7 myc/HisA

SUM 159PT GFP
SUM 159PT V5/HisA
SUM159PT myc/HisA

A publication describing these cell lines is in preparation (see manuscript by Wang et al., above). In addition, three other research groups have asked for one these cell lines (SUM 159PT GFP) for their own experiments.

5. Funding Applied For:

The preliminary data cited in this application has been used to applied for the next phase of these experiments from the USAMRMC 2000 Breast Cancer Research Program. (BC001000), and will also be incorporated into an application that will be submitted to the National Cancer Institute of the National Institutes of Health to examine the generality of these observations in other epithelial tumors, particularly of the endometrium and prostate.

Will also be incorporated into an application to the NIH/NCI Pathology B study section

LIST OF PERSONNEL BEING SUPPORTED BY THE GRANT

The following individuals have been supported by the grant, in part or in whole:

Martin Tenniswood	PI	Aug 97- Aug 98	10%
Amy Moquin	Technician	Aug 97- Aug 98	100%

After the move to University of Notre Dame, my entire salary was covered by the endowment associated with the Coleman Chair. Since Amy Moquin decided to remain in Lake Placid, Kenneth Jones was hired to replace her on the grant along with two graduate students:

Kenneth Jones	Technician	Aug 98-Jul 00	50%
Jacintha O'Sullivan	Grad Res Ass	Aug 98-Jul 00	100%
Edmund Lee	Grad Res Ass	Aug 99-Jul 00	100%

CONCLUSIONS

The experiments described in this report provides unequivocal evidence that SERMs, particularly tamoxifen, induce an invasive phenotype in MCF-7 cells *in vitro*. By itself this is an interesting phenomenon, however if these observations do not translate to the *in vivo* setting, they remain an interesting, but clinically irrelevant oddity. The genetically tagged cell lines we have developed will allow us to extend our observations into a preclinical model of breast cancer. If these studies confirm the earlier *in vitro* observations and establish that SERMs such as tamoxifen do indeed accelerate metastatic progression in a preclinical model of breast cancer, this will have very significant implications both for the use of SERMs for the treatment of primary, organ confined breast cancer and for chemoprevention.

REFERENCES

Van Weelden, K., Flanagan, L., Binderup, L., Tenniswood, M and Welsh J. (1998) Apoptotic Regression of MCF-7 xenografts in nude mice treated with the Vitamin D₃ analog EB1089. *Endocrinology* 139: 2102-2110.



DEPARTMENT OF THE ARMY

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FORT DETRICK, MARYLAND 21702-5000

*Rec'd
10/29/2001*

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure